

ETIOLOGY AND PATHOGENESIS OF PARKINSON'S DISEASE

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KEY WORDS: dopamine neurons, substantia nigra, oxidant stress, apoptosis, neuroprotection

ABSTRACT

Parkinson's disease (PD) is an age-related neurodegenerative disorder that affects approximately 1 million persons in the United States. It is characterized by resting tremor, rigidity, bradykinesia or slowness, gait disturbance, and postural instability. Pathological features include degeneration of dopaminergic neurons in the substantia nigra pars compacta coupled with intracytoplasmic inclusions known as Lewy bodies. Neurodegeneration and Lewy bodies can also be found in the locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and central and peripheral components of the autonomic nervous system. Current treatment consists of a dopamine replacement strategy using primarily the dopamine precursor levodopa. While levodopa provides benefit to virtually all PD patients, after 5–10 years of treatment the majority of patients develop adverse events in the form of dyskinesia (involuntary movements) and fluctuations in motor response. Further, disease progression is associated with the development of dementia, autonomic dysfunction, and postural instability, which do not respond to levodopa therapy. Accordingly, research efforts have been directed toward understanding the etiology and pathogenesis of PD in the hope of developing a more effective therapy that will slow or halt the natural progression of PD. This paper reviews recent advances.

ETIOLOGY

Environmental Factors

The specific etiology of Parkinson's disease (PD) is not known. Epidemiologic studies indicate that a number of factors may increase the risk of developing PD (reviewed in Tanner & Langston 1990). These include exposure to well

water, pesticides, herbicides, industrial chemicals, wood pulp mills, farming, and living in a rural environment. A number of exogenous toxins have been associated with the development of parkinsonism, including trace metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide. There has also been interest in the possible role of endogenous toxins such as tetrahydroisoquinolines and beta-carbolines. However, no specific toxin has been found in the brain of PD patients, and in many instances the parkinsonism seen in association with toxins is not that of typical Lewy body PD. The most compelling evidence for an environmental factor in PD relates to the toxin 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP). MPTP is a byproduct of the illicit manufacture of a synthetic meperidine derivative. Drug addicts who took MPTP developed a syndrome that strikingly resembled PD, both clinically and pathologically (Langston et al 1983). MPTP induces toxicity through its conversion in astrocytes to the pyridinium ion (MPP⁺) in a reaction catalyzed by monooxidase type B (MAO-B) (Singer et al 1987). MPP⁺ is then taken up by dopamine neurons and causes a mitochondrial complex I defect similar to that found in PD (Nicklas et al 1985). This observation supports the possibility that an environmental factor might cause PD; however, no MPTP-like factor has been identified in PD patients to date.

Genetic Factors

There has been considerable interest in the potential role of genetic factors in the etiology of PD (Golbe 1990). Approximately 5–10% of PD patients have a familial form of parkinsonism with an autosomal-dominant pattern of inheritance. Large pedigrees have been identified where members in different generations suffer from PD. In addition, the incidence of PD is greater in family members than in age-matched controls (reviewed in Wood 1998). The VA twin study revealed no difference in concordance between monozygotic and dizygotic twins of PD patients aged 60 years or older but a significantly increased incidence was observed in monozygotic twins who developed PD at less than 50 years of age (Tanner et al 1997). This suggests that genetic factors are important in young-onset patients but are not likely to play a major role in patients with sporadic PD. A number of candidate genes have been screened and found not to be associated with an increased risk of PD. These include Apo-ε4, tyrosine hydroxylase, glutathione peroxidase, catalase, superoxide dismutase (SOD)-1 and 2, and the dopamine D2, D3, and D4 receptors (reviewed in Gasser et al 1994). Some but not all studies have detected polymorphisms in the genes encoding CYP2D6 and MAO-A and -B. However, these gene markers account for only a small number of PD cases at most, as the allelic frequencies are relatively uncommon. More recently, an association has been demonstrated between PD and the presence of a slow acetylator phenotype

(Bandmann et al 1997). This association was stronger in patients with familial rather than sporadic PD, suggesting the possibility of increased gene loading. If validated, it warrants examination of toxins that are metabolized by N-acetyltransferase-2.

There has been an extensive search for a mutation in the mitochondrial genome, based on the finding of a defect in mitochondrial complex I in the substantia nigra pars compacta (SNc) of PD patients. Complex I is composed of 41 subunits, 7 of which are encoded by mitochondrial DNA (mtDNA). Mitochondrial DNA is a circular double-stranded molecule that is much more likely to undergo mutation than nuclear DNA is. In one study the mitochondrial genome was normal (Lestienne et al 1990). In another, a 5-kb deletion was detected, but it was identical to that found in a normal aging population (Ikebe et al 1995). Ikebe et al (1995) sequenced total mitochondrial DNA in five sporadic PD patients and noted different point mutations in a subunit of complex I in each. However, no disease-specific mutation was identified, and none had a familial form of PD. Mitochondrial DNA mutations can be maternally transmitted, but most studies have failed to detect a maternal pattern of inheritance in PD. This does not exclude mtDNA involvement, as the majority of patients with a mitochondrial mutation (e.g. deletions and the A3243G mutation) do not have a positive family history.

Recently, PD was linked to the q21-23 region of chromosome 4 in a large Italian-American family known as the Contursi kindred (Polymeropoulos et al 1996). Patients had a relatively early age of onset but otherwise demonstrated typical clinical and pathological features of PD, including Lewy bodies. Subsequently, a mutation was detected in the gene that encodes for the protein α -synuclein in this family as well as in several apparently unrelated Greek families (Polymeropoulos et al 1997). Sequence analysis demonstrated that the mutation consisted of a single base pair change from G to A at position 209 (G209A), resulting in an alanine to threonine substitution at position 53 (Ala53Thr) in the α -synuclein protein. In the affected families, 85% of patients who expressed the mutant gene had clinical features of PD, whereas this mutation was not seen in any of 314 controls. A second mutation in the α -synuclein protein (Ala30Pro) has recently been described in a German family (Krüger et al 1998). These findings provide strong evidence that a single mutation in the human α -synuclein gene is sufficient to account for the PD phenotype.

α -Synuclein is a small protein of 140 amino acids that was first identified in the Pacific electric ray, *Torpedo californica* (Maroteaux et al 1988). Interestingly, the Ala53Thr mutated form of the human α -synuclein protein is normally expressed in zebra fish and mice. In humans, a fragment of the α -synuclein protein known as the non-beta amyloid component (NAC) has been isolated from senile plaques in the brains of patients with Alzheimer's

disease (AD) (Ueda et al 1993). It has now been determined that NAC derives from non-amyloid component precursor protein (NACP), which is homologous to α -synuclein. Mutations in the α -synuclein gene have not been identified in patients with sporadic PD, but immunocytochemistry has demonstrated that α -synuclein is an abundant component of Lewy bodies, even in patients with familial or sporadic PD who do not have the gene mutation (Spillantini et al 1997). This suggests that accumulation of α -synuclein may be central to the development of PD.

Little is known about the neurobiology of α -synuclein. An avian homolog of the synucleins, synelfin, is up-regulated during a critical period of song learning, suggesting that it may play a role in plasticity (George et al 1995). The physicochemical properties of the protein suggest that it is a natively unfolded molecule that can self-aggregate and form amyloid fibrils (Weinreb et al 1996). It has been proposed that the gene mutation may alter the structure of α -synuclein, making it more prone to self-aggregation and therefore difficult to destroy by proteasomes (Polymeropoulos 1998). Alternatively, it can be proposed that a primary proteasomal defect or oxidant damage to proteins sufficient to prevent their clearance by normal proteasomes might account for the accumulation of nonmutated α -synuclein in patients with sporadic PD (Jenner & Olanow 1998). This concept is supported by the finding that α -synuclein co-localizes with ubiquitin, suggesting that it is being prepared for proteasomal digestion. Recent studies note that apoptosis of nigral neurons is associated with up-regulated expression of α -synuclein gene and protein (Kholodilov et al 1997). In this model, α -synuclein staining is widespread in the SNc but is confined to surviving neurons rather than those undergoing apoptosis (R Burke, personal communication). These findings may indicate that up-regulation of α -synuclein may be related to apoptosis or to the promotion of neuronal survival under conditions of stress.

The association of a PD syndrome with both MPTP and mutations in α -synuclein suggests that either an environmental or a genetic factor can cause PD. However, it is unlikely that in the majority of cases PD will be explained by a single cause. This concept has given rise to the "double hit hypothesis," which posits that PD may result from an interaction between multiple genetic mutations and/or the combination of a mutant gene and an environmental toxin. In support of this concept, we have recently shown that there is subclinical nigral degeneration in the SOD-mutant mouse and that dopamine neurons in this mouse are highly sensitive to small doses of MPTP that do not affect the wild-type littermate (Good et al 1997). Although it is unlikely that an α -synuclein mutation will account for many cases of PD, this discovery may permit the development of a transgenic animal and provide an opportunity to better understand the mechanism of cell death in PD.

PATHOGENESIS

Oxidative Stress

Oxidative stress has received the most attention in PD because of the potential of the oxidative metabolism of dopamine to yield hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) (reviewed in Halliwell & Gutteridge 1985; Olanow 1990, 1993) (Figure 1). Oxidant stress and consequent cell death could develop in the SNc under circumstances in which there is (a) increased dopamine turnover, resulting in excess peroxide formation; (b) a deficiency in glutathione (GSH), thereby diminishing the brain's capacity to clear H_2O_2 ; or (c) an increase in reactive iron, which can promote OH^\bullet formation. Indeed, postmortem studies in PD brains demonstrate increased iron, decreased GSH, and oxidative damage to lipids, proteins, and DNA, suggesting that the SNc is in a state of oxidant stress (reviewed in Jenner & Olanow 1996).

IRON Numerous studies, using a variety of analytical techniques, have demonstrated that iron levels are increased within the substantia nigra of PD patients (Dexter et al 1989a, reviewed in Olanow & Youdim 1996). Laser microprobe (LAMMA) studies indicate that iron accumulates primarily within neuromelanin granules of dopaminergic neurons (Good et al 1992). Antibodies to specific neuronal isoforms of ferritin do not show evidence of a compensatory increase (Connor et al 1995), suggesting that the iron may be unbound and in a reactive form. Infusion of iron into the SNc of rodents induces a model of PD characterized by a concentration-dependent and progressive loss of striatal dopamine, degeneration of SNc neurons, and behavioral changes (Sengstock

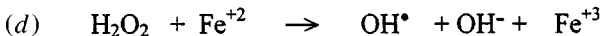
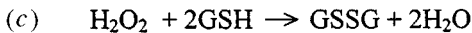
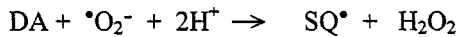
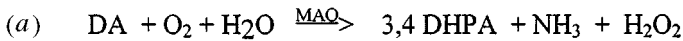


Figure 1 Both the enzymatic and the chemical metabolism of dopamine result in the formation of hydrogen peroxide (H_2O_2) (a and b). H_2O_2 is normally cleared by reduced glutathione (GSH) (c). However, an increase in the steady-state concentration of H_2O_2 can lead to a reaction with ferrous iron that generates the highly reactive and potentially cytotoxic hydroxyl radical (OH^\bullet) according to the Fenton reaction (d).

et al 1993, 1994). How iron accumulates within the SNc in PD is not known. Increased lactoferrin receptors have been detected on nigral neurons in PD patients and might account for preferential accumulation of iron within these cells (Faucheux et al 1995). It is not clear if iron accumulation in PD is primary or secondary. Iron accumulation in affected areas can be seen in a variety of other neurodegenerative conditions. Further, increased SNc iron has been observed following MPTP treatment or 6-hydroxydopamine (6-OHDA) lesions (Temlett et al 1994, Oestreicher et al 1994). These findings indicate that iron can accumulate secondary to cell degeneration from a variety of causes. However, this does not negate its potential importance in PD, as iron can still contribute to cell death even if it accumulates secondary to another cause.

GLUTATHIONE A defect in one or more of the naturally occurring antioxidant defenses could lead to neurodegeneration in PD (reviewed in Jenner & Olanow 1996). No basic defects have been detected in levels of ascorbic acid, α -tocopherol, catalase, or glutathione peroxidase. Mn-SOD activity is elevated, consistent with an adaptive increase in the inducible form of the enzyme. Most attention has been directed to the finding of a selective decrease in the reduced form of glutathione (GSH) in the SNc in PD (Sofic et al 1992, Sian et al 1994a). Reduced levels of GSH have not been detected in other brain areas in PD and have not been reported in any other degenerative disorder. A reduction in GSH may impair H_2O_2 clearance and promote OH^\cdot formation, particularly in the presence of increased iron. The cause of the decrease in GSH in PD is unknown. There are no defects in the major enzymes associated with glutathione synthesis. There is, however, a significant increase in the level of γ -glutamyltranspeptidase (γ -GTT), the enzyme responsible for the translocation of glutathione precursors and metabolism of the oxidized form of glutathione (GSSG) (Sian et al 1994b). Increased γ -GTT may reflect an attempt by surviving cells to recruit glutathione precursors into the cell to replenish diminished levels of GSH or a compensatory mechanism to remove potentially toxic GSSG formed as a consequence of oxidant stress.

A defect in GSH, comparable to that found in PD, has been detected in the nigra of patients discovered at autopsy to have incidental Lewy bodies (ILB) and thought to have preclinical PD (Dexter et al 1994a). As changes were not detected in iron, mitochondrial complex I, or other markers of oxidant stress, this suggests that a decrease in GSH may represent the initial biochemical defect in PD. We have demonstrated that buthionine sulfoximine (BSO), a selective inhibitor of α -glutamylcysteine synthetase, induces a reduction in GSH and is toxic to cultured dopaminergic neurons, but only when levels have been depleted by 80% (Mytilineou et al 1998), possibly because GSH is preferentially conserved within mitochondria. In rats, administration of BSO sufficient to

induce a 40–60% decline in GSH, paralleling the degree of loss that occurs in PD, does not affect the number of tyrosine hydroxylase (TH)-positive cells in the SNc (Toffa et al 1997). However, this level of GSH depletion enhances the neurodegeneration that is observed when the rodents are treated with other toxins such as 6-OHDA or MPP⁺ (Pileblad et al 1989, Wüllner et al 1996). These observations suggest that a reduction in GSH by itself may not damage dopamine neurons but may render them vulnerable to other toxins.

OXIDATIVE DAMAGE There is evidence of oxidative damage in the brains of PD patients. Increased levels of the lipid peroxidation products malondialdehyde (MDA) and lipid hydroperoxide have been found in the SNc but not the cerebellum of PD patients (Dexter et al 1989b, 1994b). Increased staining for 4-hydroxynonenal, a product of lipid peroxidation that has the capacity to alter proteins and promote cell toxicity, has been detected in surviving dopaminergic neurons (Yoritaka et al 1996). Additionally, increased levels of protein carbonyls and 8-hydroxy-2-deoxyguanosine, reflecting oxidative damage to proteins and DNA, respectively, have been found in the SNc as well as in numerous other brain regions of PD patients (Alam et al 1997a, Sanchez-Ramos et al 1994, Alam et al 1997b). Overall, these results suggest that there is widespread oxidative damage in PD. However, the majority of PD patients receive levodopa therapy, and it is uncertain if its oxidative metabolites contribute to the oxidative damage detected postmortem. Levodopa has been shown to induce degeneration of cultured dopamine neurons (Mytilineou et al 1993, Walkinshaw & Waters 1995) but has not been shown to be toxic to dopamine neurons in normal rodents and humans. The situation may be different in PD where defense mechanisms are impaired. Indeed, levodopa can augment neuronal degeneration and increase lipid peroxidation in rodents pretreated with 6-hydroxydopamine (Ogawa et al 1994). It remains to be determined if the oxidative damage that occurs in PD is a primary event or occurs secondary to an alternate etiology, drugs, or postmortem events.

Mitochondrial Dysfunction

A selective 30–40% decrease in complex I activity of the mitochondrial respiratory chain has been found in the SNc of PD patients (Schapira et al 1990). Other brain regions are unaffected, and a similar defect has not been found in patients with multiple system atrophy (MSA) who have also experienced extensive degeneration of nigrostriatal neurons and been exposed to levodopa. A complex I defect has also been found in platelets and muscle of PD patients, but these results are less consistent, especially in muscle (DiMauro 1993). The cause of the decreased complex I activity in PD remains a mystery. MPTP-like toxins have not been detected. No specific abnormality has been detected in

the subunits of complex I or in the mitochondrial or nuclear genes that encode complex I proteins. In a recent study, a complex I defect was found in cybrids carrying mtDNA derived from PD platelets (Swerdlow et al 1996). This indicates the presence of a defect in the mitochondrial genome that can be transferred through multiple passages. Such a defect could be due to an inherited mutation or to a toxic insult, possibly secondary to oxidant stress, although disease-related mtDNA mutations have not been detected in PD and oxidative stress typically affects both complexes I and IV.

A mitochondrial complex I defect could contribute to cell degeneration in PD through decreased ATP synthesis and a bioenergetic defect (see below). In mouse brain synaptosomes, complex I inhibition by MPTP or MPP⁺ can lead to depletion of cellular ATP (Scotcher et al 1990). However, studies in experimental animals indicate that a decrease in complex I activity of 40% or less does not compromise cellular ATP levels (Davey & Clark 1996). A decrease in α -ketoglutarate dehydrogenase (α -KG) immunostaining has also been detected in PD (Mizuno et al 1994). The combination of a decrease in both α -KG and complex I activity would be more likely to adversely affect cellular energy metabolism than would a defect in either enzyme alone.

A mitochondrial complex I defect could also lead to cell damage through free radicals generated directly at this site or by way of a compensatory increase in respiration at complex II. It is noteworthy that MPTP toxicity can be attenuated by free radical scavengers and by coenzyme Q, a redox component of the mitochondrial respiratory chain that accepts electrons from complex I or II (Schulz et al 1995a). A complex I defect might also contribute to the development of apoptosis. Increasing evidence suggests that a reduction in the mitochondrial membrane potential as a result of impaired proton pumping can lead to opening of a mitochondrial permeability transition pore and the release of small mitochondrial proteins that signal for the onset of apoptosis (see section below for details). As complex I is the major site of proton pumping, it is possible that a complex I defect in PD may contribute to neuronal vulnerability and lead to apoptosis.

Excitotoxicity

Excitotoxicity is an established cause of neurodegeneration that has been implicated in PD based on two possible mechanisms. The first involves "strong" excitotoxicity resulting from increased glutamate formation. SNc dopaminergic neurons are rich in glutamate receptors, receive extensive glutamate innervation from the cortex and the subthalamic nucleus (STN), and demonstrate a pattern of burst firing in response to exogenously administered glutamate (Rothstein et al 1994, Johnson et al 1992). Dopamine lesions disinhibit the STN and increase the firing rate of its excitatory output neurons (DeLong 1990). It can

be postulated that as STN fibers project to the SNc, dopamine lesions promote further excitotoxic damage (Rodriguez et al 1998). Indeed, STN lesions protect nigral neurons from 6-OHDA toxicity (Piallat et al 1995). A second hypothesis involves the "weak" excitotoxic mechanism (Beal 1992). This theory suggests that a reduction in energy metabolism due to a defect in mitochondrial function results in a loss of the ATP-dependent Mg-blockade of N-methyl-D-aspartate (NMDA) receptors and allows physiological concentrations of glutamate to mediate a calcium influx into the cell. In support of a role of excitotoxicity in PD are reports that NMDA antagonists protect against dopamine cell loss resulting from MPP⁺ infusion into the SNc of rats (Turski et al 1991) and MPTP treatment in primates (Greenmayre et al 1994).

Excitotoxic damage is thought to be mediated, at least in part, via nitric oxide (NO) (Dawson et al 1991). NO is formed by the conversion of arginine to citrulline in a reaction catalyzed by nitric oxide synthase (NOS). A glutamate-mediated rise in cytosolic calcium results in activation of NOS with increased NO production. NO reacts with superoxide radical to form peroxynitrite and hydroxyl radical, both powerful oxidizing agents (Beckman et al 1990). NO might also contribute to cell degeneration by displacing iron from binding sites on ferritin, so that it can participate in the Fenton reaction, and by inhibiting mitochondrial complex IV, thereby potentially converting a reversible complex I defect into an irreversible respiratory chain defect. Indeed, Bolanos et al (1996) recently demonstrated that the mitochondrial respiratory chain is damaged by sustained exposure to NO and that GSH is an important defense. This has implications for PD where GSH levels are decreased. NO-mediated toxicity has been implicated in nigral damage induced by MPTP. The neuronal NOS inhibitor 7-nitroindazole (7-NI), which blocks NO formation, protects dopaminergic neurons from MPTP toxicity in both rats and baboons (Schulz et al 1995b, Hantrave 1996). Similarly, MPTP toxicity is diminished in NOS knock-out mice (Przedborski et al 1996). A recent report noted that 7-NI inhibits MAO-B (Castagnoli et al 1997), raising the possibility that it may act by blocking the conversion of MPTP to MPP⁺. However, recent studies indicate that 7-NI is also effective in blocking MPP⁺ toxicity (F Beal, personal communication). Damage due to NO can be estimated by measuring the formation of 3-nitrotyrosine (3-NT), a product of the peroxynitrite-induced nitration of tyrosine residues on cellular proteins (Ischiropoulos et al 1992). Increased levels of 3-NT have been reported in MPTP-treated mice and monkeys (Schulz et al 1995a). We have also demonstrated increased 3-NT immunostaining in the core of Lewy bodies in PD patients (Good et al 1998). The presence of increased 3-NT staining in PD is consistent with the notion that increased NO formation and peroxynitrite contribute to cell damage in PD.

Neurotrophic Factors

The classic *in vitro* studies of Levi-Montalcini & Hamburger (1953) showed that cultured sympathetic neurons could not survive if deprived of nerve growth factor (NGF). Similarly, axotomy can induce degeneration of nigrostriatal dopamine neurons by depriving them of access to essential survival factors in target tissues (Hagg & Varon 1993). It is also apparent that both nerve cells and astrocytes can synthesize mRNAs and protein for a variety of neurotrophic molecules, including ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF), that have the capacity to support the survival of neighboring nerve cells. In the normal adult central nervous system, these trophic factors are constitutively expressed at low levels, but they can be up-regulated following injury. Sublethal neuronal damage in adult rats induces reactive astrocytes with up-regulation of neurotrophic factors such as CNTF, NGF, and fibroblast growth factor (FGF) (Chadi et al 1994, Asada et al 1995). Reactive astrocytes have been demonstrated in regions of dopaminergic nerve cell loss in PD (McGeer et al 1988). There is also strong evidence that a number of trophic molecules have the capacity to protect dopamine neurons from toxic insult. BDNF increases survival of cultured dopaminergic neurons and protects them from exposure to MPTP (Hyman et al 1991). Both GDNF and CNTF protect SNc neurons in rats from transection of nigrostriatal axons (Lin et al 1993, Hagg et al 1992). GDNF has been shown to increase the survival and sprouting of dopaminergic neurons in dopamine-lesioned rodents and primates (Tomac et al 1995, Gash et al 1995) and to reverse parkinsonian features in MPTP-treated primates (Lapchak et al 1997). Both neuroprotective and neurorestorative benefits have been seen (Gash et al 1998). An *in situ* hybridization study found no detectable levels of GDNF mRNA in brains obtained from PD patients or age-matched controls (Hunot et al 1996). It would seem likely, therefore, that a decline in GDNF expression does not initiate dopaminergic cell loss. However, a reduction in the capacity to up-regulate trophic factors in response to injury might remove an important defense mechanism and contribute to cell degeneration. Alternatively, administration of trophic factors may rescue or protect dopamine neurons. Systemic delivery of trophic factors is limited by central nervous system penetrance, and effective therapy in animal models requires direct intracerebral or intrathecal delivery. A small trial of intraventricular GDNF has been initiated in PD patients, but results are not yet available. Other possible approaches include the use of implanted or encapsulated cells that express trophic factors or adenovirus vectors that transfect cells in the SNc or striatum to produce a trophic factor. Preliminary studies administering GDNF by each of these methods have been shown to protect dopamine cells in rodents, illustrating their potential value in PD (Gash et al 1998).

Glial Immune Modulators

Microglia and cytokines are known to modulate cellular responses after injury to the central nervous system. Large numbers of reactive (HLA-DR) positive microglia have been observed in the SNc in PD, particularly in areas of maximal neurodegeneration, namely the ventral and lateral portion of the SNc (McGeer et al 1988). Levels of interleukin-1 β (IL-1 β), interferon- γ (INF- γ), and tumor necrosis factor- α (TNF- α) in the SNc of PD patients are increased by 760–1570% in comparison with normal controls (reviewed in Hirsch et al 1998). TNF- α is particularly increased in PD in the region of neuromelanin-containing neurons or debris. Activation of TNF- α receptors is associated with nuclear translocation of NF κ -B and with the development of apoptosis in cultured dopaminergic neurons (Hunot et al 1997). Interestingly, NF κ -B translocation and apoptosis are preceded by the transient production of free radicals, and these events can be prevented by the antioxidant N-acetyl cysteine, indicating that an oxidant-mediated apoptogenic transduction pathway may play a role in the neuronal death. In PD patients, there is a 70-fold increase in nuclear translocation of NF κ -B, suggesting that activation of TNF- α and similar transduction mechanisms may play a similar role in the neurodegeneration that occurs in this condition.

An immune model of PD has been created through the use of antibodies generated against a hybrid line of dopaminergic neurons (Crawford et al 1992). The animals exhibited hypokinesia and a significant loss of SNc neurons, raising the possibility that immune mechanisms could contribute to human PD. Epidemiologic studies suggest that anti-inflammatory agents such as aspirin and steroids may protect against the development of AD (McGeer et al 1996), possibly through blockade of NF- κ B activation (Grilli et al 1996). Such studies have prompted trials of anti-inflammatory agents in neurodegeneration. However, a pilot study of prednisone in AD patients did not reveal any cognitive or behavioral benefits (Aisen et al 1996). Similar trials have not yet been conducted in PD.

A newly recognized class of agent, which appears to share properties with trophic factors and immune-modulating molecules, is the immunophilins. These molecules bind to a component of the cyclosporine binding site and lack its immunosuppression properties. Immunophilin ligands prevent the loss of TH neurons and promote dopamine neurite extension following 6-OHDA or MPTP lesions in tissue culture and rodent models (Steiner et al 1997). Enhanced TH staining was observed with immunophilins in MPTP-treated mice even when the drug was administered as long as one month following the dopaminergic lesion. The mechanism of action of the immunophilin ligands is not yet clear, although one ligand, pentoxifylline, has been shown to modulate cytokine production, specifically the down-regulation of TNF- α (Okuda et al 1997).

It remains unclear whether an immune/inflammatory component is a primary or secondary event in PD or whether changes observed are due to an autoimmune process or the natural response of microglia and astroglia to neuronal damage. However, it is becoming increasingly likely that glia play some role in neurodegenerative conditions. Astrocytes have been shown to protect cultured neurons from oxidative damage induced by H_2O_2 (Desagher et al 1996). Furthermore, GSH is synthesized primarily within glia and transported to neurons in response to neural excitatory stimuli. The decrease in GSH found in the SNc in PD therefore might be secondary to a glial defect, as the magnitude of loss is more than can be accounted for by a loss of neuronal GSH alone. On the other hand, we have found that GSH depletion induces degeneration of cultured dopamine neurons only in the presence of glia, suggesting that cytokines or NO generated from glia contributed to neurodegeneration in this model (C Mytilineou, CW Olanow, unpublished data). It remains to be determined whether anti-inflammatory or trophic factor supplementation will provide effective therapy for PD patients given the inherent difficulties in providing adequate drug delivery to the CNS. A greater understanding of the role of glial cells and their regulation of neuroactive molecules might nonetheless contribute to the design of therapies that will protect or repair degenerating nerve cells.

APOPTOSIS

Apoptotic Neuronal Death

There has been increasing interest in the notion that cell death in PD occurs by way of apoptosis rather than necrosis. Necrosis is a rapid form of cell death that is characterized by (a) massive ionic fluxes (particularly Ca^{2+}) across the plasma membrane, (b) activation of Ca^{2+} -dependent proteases with widespread intracellular protein digestion, (c) mitochondrial disruption with a complete loss of ATP production, (d) massive cellular swelling with disruption of subcellular organelles and rupture of plasma membrane, (e) inflammatory response secondary to spilling of cellular contents into the extracellular space, and (f) relative preservation of nuclear DNA. In contrast, apoptosis is a gradual form of cell death that is characterized by (a) marked cell shrinkage, (b) preservation of plasma membranes, (c) absence of an inflammatory response as membrane-wrapped pieces of the cell are engulfed by macrophages, (d) cytoskeletal depolymerization, (e) fragmentation of nuclear DNA by endonucleases, and (f) chromatin condensation with the formation of nuclear or "apoptotic" bodies. Apoptosis functions as a counterbalance for excess cell replication and therefore initially seemed unlikely to involve mature nerve cells which do not normally have the capacity to replicate. It is now appreciated that neuronal apoptosis can result from a variety of insults, many of which may be relevant to the

pathogenesis of PD. These include levodopa, dopamine, iron, glutathione depletion, excitatory amino acids, MPTP, MPP⁺, 6-hydroxydopamine, mitochondrial complex I inhibitors, and pro-oxidants (reviewed in WG Tatton et al 1997). In general, low concentrations of a toxin, particularly when delivered slowly, induce apoptosis, while high levels or rapid delivery of the same toxin induces necrosis.

A number of genes and their protein products are known to influence apoptosis (see Bredesen 1995, Kroemer et al 1995). In nerve cell apoptosis, the bax/bcl family (bax, bcl-2, bcl-x_L) and the interleukin 1 β converting enzyme (ICE) family (ice, ich-1_L, and ich-1_S) or caspases have received particular attention. Increased expression of bax or caspase promotes apoptosis, whereas increased expression of bcl-2, bcl-x_L, and ich-1_S promotes survival. Two other gene/proteins have been shown to strongly influence neuronal apoptosis. The early gene *c-jun* is transiently expressed in the early stages of neuronal apoptosis. Antisense oligonucleotides that block the translation of *c-jun* mRNA and overexpression of a negative *c-jun* mutant reduce apoptosis and facilitate neuronal survival (Schlingensiepen et al 1994). In contrast, overexpression of *c-jun* increases apoptosis (Ham et al 1995). Similarly, overexpression of Cu/Zn superoxide dismutase (SOD-1) decreases neuronal apoptosis, whereas apoptosis is increased when SOD-1 is underexpressed (Rothstein et al 1994, Troy & Shelanski 1994).

Apoptosis and Mitochondria

It is now appreciated that mitochondria are critical to some forms of apoptosis (Tatton & Olanow 1998). This is illustrated by the finding that mitochondrial homogenates can induce nuclear changes characteristic of apoptosis in cell-free systems (Newmeyer et al 1994). Mitochondrial complexes I, III, and IV of the respiratory chain pump protons out of the mitochondrial matrix across the inner mitochondrial membrane using electron energy provided by the carrier molecules nicotinamide adenine dinucleotide (NADH), ubiquinone, and cytochrome C (Cyt C). This is reflected by a voltage difference across the inner mitochondrial membrane, termed the mitochondrial membrane potential ($\Delta\Psi_M$). A fall in $\Delta\Psi_M$ coupled with an increase in intramitochondrial calcium is associated with opening of a mitochondrial megapore, known as the permeability transition pore (PTP), and the release of apoptosis initiating factors (AIFs), such as cytochrome C or an ICE-like protease that signal for the initiation of apoptosis (Liu et al 1996, Susin et al 1996a). The PTP spans the inner and outer mitochondrial membranes and is comprised of an adenine nucleotide translocator (AdNT), a voltage-dependent anion channel (a porin), and a peripheral benzodiazepine binding protein (Zoratti & Szabo 1995). Factors like glutathione, ADP, and ROS in the mitochondrial matrix modulate the gating voltages necessary to induce PTP opening. Opening of the pore allows free exchange of solutes and

small proteins between the mitochondrial matrix and the extramitochondrial cytosol. Mitochondrial AIFs may be released directly through the PTP or through fractures that develop in the mitochondrial membrane. Agents that maintain closure of the PTP, such as BCL-2 or cyclosporine A, prevent the fall in $\Delta\Psi_M$ and the release of AIFs and are thereby antiapoptotic (Susin et al 1996b). Measurements in a variety of blood, hepatic, and immune cell models have shown that $\Delta\Psi_M$ is reduced early in the apoptotic process, prior to the onset of nuclear DNA fragmentation and chromatin condensation (Susin et al 1996b). Using laser confocal microscopy, our group has extended these findings to a neuronal model of apoptosis and established that $\Delta\Psi_M$ decreases prior to nuclear DNA fragmentation (Wadia et al 1998). In our studies, the decrease in $\Delta\Psi_M$ correlated temporally with a rise in intramitochondrial Ca^{2+} and anteceded a rise in cytosolic free radicals, suggesting that the latter is likely a secondary event.

Apoptosis and Parkinson's Disease

There have been several reports of apoptosis in postmortem PD brains (Agid 1995, Mochizuki et al 1996, Anglade et al 1997, N Tatton et al 1998), as there have been in other neurodegenerative diseases such as AD, amyotrophic lateral sclerosis, and Huntington's disease (reviewed in WG Tatton et al 1997, Olanow et al 1998). The key identifying features of apoptosis involve nuclear DNA. Endonucleases activated in the late stages of apoptosis cleave nuclear DNA into pieces of varying length such that DNA electrophoresis may reveal a repeating "ladder" pattern. DNA gel electrophoresis has been used to detect DNA digestion; however, it requires fragmented DNA from large numbers of cells and is therefore appropriate only when thousands of cells enter apoptosis in a synchronized manner. As degenerating nerve cells in PD likely enter apoptosis in a desynchronized manner over a prolonged period of time, and the life span of nuclei with fragmented DNA is probably only a matter of hours, electrophoresis is unlikely to detect the small numbers of cells undergoing apoptosis in PD at a single point in time. Electron microscopy was used in one study to detect features of apoptosis in PD (Anglade et al 1997), but it is a time-consuming and impractical method for quantitating apoptosis in specific regions of the brain. Two other methods have been used to detect small numbers of apoptotic nuclei in PD: (a) *in situ* 3'-end labeling (ISEL) techniques such as the ApopTag, TUNEL, or BODIPY/Florescein dUTP, which attach a chromagen or a fluorochrome to the cut ends of nuclear DNA, and (b) fluorescent DNA binding dyes that label regions of chromatin clumping and apoptotic bodies.

ISEL techniques have been used primarily to provide evidence for apoptosis in neurodegenerative conditions. In PD, approximately 1–2% of SNc neurons had ISEL-positive nuclei (Agid 1995, Mochizuki et al 1996). These percentages seem high, given the short life span of nuclei with detectable DNA strand

breaks and the likelihood that nerve cell death occurs asynchronously over a number of years in PD. Tatton & Kish (1997) used a combination of an ISEL technique and concurrent staining for chromatin condensation with acridine orange to unambiguously establish that SNc neurons in the mouse had entered apoptosis following MPTP administration. In this model, where cell death occurs over 5–10 days, ISEL-positive nuclei were seen in a maximum of 6–10% of SNc dopaminergic neurons on day five and fell to less than 1% at later time points (Tatton & Kish 1997). MPTP damage to dopaminergic neurons is likely to be much more synchronized than occurs in PD, so it seems unlikely that comparable percentages of apoptotic cells will be found in PD patients. This has raised concern that ISEL methods may overestimate the number of apoptotic nuclei and that staining may be related to postmortem events. However, in PD studies, increased numbers of ISEL-positive nuclei were found only in the SNc. They were not detected in other brain regions and were not seen in the SNc of controls. These findings are hard to explain by postmortem changes alone. Further, we have recently examined PD brains for the presence of apoptotic nuclei using both an ISEL technique and DNA staining for chromatin condensation (N Tatton et al 1998). We demonstrated the presence of apoptotic nuclei in approximately 2% of SNc melanin-containing neurons in PD patients compared with 0.2% in age-matched controls. We believe that positive results with both of these techniques in individual neurons establish the presence of apoptosis and avoid the false positive results that may occur with either one. The large number of apoptotic cells seen in the SNc of PD patients in these studies may reflect accelerated apoptosis resulting from agonal events in vulnerable neurons or in those that were already committed to undergo apoptosis. In support of this concept, alterations in BCL-2 expression have been found in surviving SNc neurons in PD brains (Mogi et al 1996). Further, we have found that mitochondrial membrane potential is reduced in cultured fibroblasts derived from some PD patients (R Chalmers-Redman, CW Olanow, WG Tatton, unpublished observations). If a decrease in mitochondrial membrane potential is also present in SNc neurons in PD, it may represent the fundamental problem in PD and account for these neurons' propensity to enter apoptosis under circumstances where normal neurons would survive. Thus, the relatively high percentage of ISEL-positive nuclei found in PD brains may be due to accelerated DNA cleavage in the immediate pre-agonal period in neurons destined to undergo apoptosis at a later date or in neurons vulnerable to agonal stresses.

THE FUTURE

Controlling Parkinson's Disease

Based on current knowledge regarding the etiology, pathogenesis, and mechanism of cell death in PD, numerous neuroprotective strategies might be devised.

Eliminating a primary etiology is most desirable, but it is unlikely to be effective in view of the probability that different environmental and genetic factors likely contribute to the development of PD and that multiple causes may be operative even in an individual patient. Neuroprotection might be provided by agents that interfere with factors involved in pathogenesis. These could include antioxidants, bioenergetics, agents that interfere with excitotoxicity or prevent a rise in cytosolic free calcium, trophic factors, and anti-inflammatory drugs. To date, most clinical trials have focused on antioxidants. A prospective, double-blind, placebo-controlled study found no advantage of taking vitamin E (Parkinson Study Group 1993). The selective MAO-B inhibitor selegiline [(–)-deprenyl] was tested based on its capacity to prevent MPTP-parkinsonism and to inhibit peroxides formed by the MAO-B oxidation of dopamine. Selegiline was shown to delay the emergence of disability and to slow the progression of signs and symptoms of PD (Parkinson Study Group 1993, Olanow et al 1995). However, there remains a question as to whether the benefits observed were related to neuroprotection or to a confounding symptomatic effect of the drug that masked underlying neurodegeneration. Clinical trials of the NMDA receptor antagonist remacemide, the glutamate antagonist riluzole, the bioenergetic agent coenzyme Q, and dopamine agonists that are thought to diminish ROS generated by dopamine turnover are currently under way, but no clinical data are available yet.

If apoptosis is confirmed in PD, it provides an opportunity to interfere with neuronal death. This might be accomplished by agents that maintain closure of the PTP, preserve $\Delta\Psi_M$, and prevent release of AIFs, as well as by agents that up-regulate the synthesis of antioxidant and antiapoptotic molecules, such as SOD-1, GSH, and BCL-2. Because such treatments involve the effector stage of apoptosis, they may have the advantage of providing benefit to patients regardless of the specific etiology or pathogenesis of PD. There is now evidence that selegiline neuroprotection is related to inhibition of apoptosis and not to MAO-B inhibition (Tatton & Chalmers-Redman 1996, Mytilineou et al 1997a). Selegiline has been shown to protect dopamine neurons in a variety of model systems. Benefit is dependent on transcriptionally mediated new protein synthesis and is associated with up-regulation of a number of antiapoptotic molecules, including SOD-1, glutathione, BCL₂, and BCL_{XL} (WG Tatton et al 1996, Mytilineou et al 1998). Further, selegiline prevents the loss of $\Delta\Psi_M$ in neuronally differentiated PC-12 cells deprived of serum and NGF (Wadia et al 1998). New data indicate that selegiline derives its protective benefit from its metabolite, desmethyl selegiline (DMS) (Mytilineou et al 1997b, 1998). This finding has important clinical implications; for example, in laboratory studies, higher doses are associated with greater levels of neuroprotection. However, in humans, selegiline at doses greater than 10 mg per day is a nonselective MAO

inhibitor with the potential to induce a fatal hypertensive reaction known as the "cheese effect." For this reason, doses of selegiline higher than 10 mg per day have not been employed in PD. The situation may be different with DMS, as it has a reduced capacity to inhibit MAO and is therefore likely to be better tolerated in high doses in PD patients.

There is also evidence indicating that DMS acts by binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an intermediary enzyme in glycolytic metabolism (K Borden, G Carlile, WG Tatton, unpublished observations). GAPDH normally exists as a tetramer and is located in the cytoplasm, bound to adenine-uracil (AU)-rich regions of RNA. Translocation of GAPDH to the nucleus has been shown to be associated with apoptosis (Ishitani et al 1996, Sawa et al 1997). Through the use of confocal laser microscopy and size exclusion chromatography, it has been shown that DMS preferentially maintains GAPDH as a dimer, in which form it does not accumulate in the nucleus and does not promote apoptosis (G Carlile, K Borden, R Chalmers-Redman, WG Tatton, unpublished observations). Delineation of the relationship between DMS, GAPDH, and apoptosis will hopefully point the way to the development of newer, more potent agents that can slow the progression of PD by protecting vulnerable neurons and reducing the death of dopaminergic neurons. Clinical trials in PD of putative antiapoptotic drugs such as DMS are anxiously awaited. Ultimately, it may be that combined approaches that interfere with components of both the pathogenic and apoptotic pathways will be necessary to provide neuroprotection. The recent identification of a gene that causes a PD phenotype may provide an essential clue in determining which factors are the most relevant to cell death in PD and a model in which to test putative neuroprotective agents.

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